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Characterisation of mutations and genotype–phenotype correlation in cystic fibrosis: Experience from India

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Abstract

Background: Very little is known about the genetics of cystic fibrosis (CF) from the Indian subcontinent. The aims of the study were to identify the mutations and study the relation of genotype with phenotype in Indian children with CF.
Methods: A total of 100 patients with CF were screened for mutations in the *CFTR* gene. These included c.1521_1523delCTT (p.F508del) and c.3849+10 kb C>T mutations followed by single strand conformation polymorphism/heteroduplex analysis for mutations in 19 out of 27 exons of the *CFTR* gene.
Results: At least one mutation was identified in 40 patients. The most common mutation identified was p.F508del; 20 patients were homozygous and 13 heterozygous. In addition, c.3849+10 kb C>T, c.1161delC, and p.S549N were identified in two patients each and p.R352Q, p.R1158X and p.R75Q were identified in one patient each. Three novel mutations, viz. c.1002-7_1002-5delTTT, p.G149X and p.L183I were also identified. Majority of patients who were p.F508del positive originated from Pakistan and north-western states of India. The phenotypes of all patients were classical. Genotype–phenotype correlation revealed that p.F508del positive patients had a more severe disease, manifesting at an earlier age.
Conclusions: A strategy for mutation screening for CF in India must involve testing for p.F508del followed by c.1161delC, c.3849+10 kb C>T and p.S549N. There is a need for large multicentric studies using more sensitive techniques for the identification of mutations in Indian CF patients.

Keywords: Cystic fibrosis; India; Mutations; Genotype–phenotype correlation

1. Introduction

Cystic fibrosis (CF) is a multisystem disorder caused by a defect in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene located on the long arm of chromosome 7. CF is the most common fatal genetic disorder among Caucasians, with an incidence of approximately 1 in 2000 to 4000 births, and is relatively less common among Africans and Asians [1]. More than 1300 mutations in the *CFTR* gene have been reported to the Cystic Fibrosis Genetic Analysis Consortium (CFGAC) (http://www.genet.sickkids.on.ca).

CF has been reported from India since the 1960s, but the precise prevalence rate is still unknown [2–4]. The estimated prevalence of CF among Indians living in UK and USA is 1 in 10,000 to 1 in 12,000 and 1 in 40,000 respectively [5–7]. The prevalence of CF in a north Indian population was estimated to be 1 in 43,321 to 1 in 100,323 by screening 955 cord blood samples for the presence of p.F508del mutation

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[8]. The preliminary reports on the mutation spectrum in Indian CF children indicate that p.F508del is the most common mutation, accounting for about 19–40% of the mutations [9–12]. Identification of the mutations is of great importance for further evaluation, patient counselling and prenatal diagnosis [13]. In this study, we aimed to identify the mutation spectrum in Indian patients with CF, which would help us in forming a strategy for DNA-based diagnosis, and to compare the genotypes with phenotypes.

2. Materials and methods

The present study was carried out as a descriptive pilot study on 100 patients at the Genetics Unit of Department of Paediatrics at All India Institute of Medical Sciences, New Delhi, India over a period of 5 years from 1999 to 2004. The study population included patients attending the paediatric outpatient clinics (paediatric chest and genetics clinics) and those who were admitted in the paediatric medicine or surgery wards with a suspected diagnosis of CF. Patients with sweat chloride values of >60 mEq/L on two or more occasions were included in the study. All children were scored on a scale of 5 to 25 based on their nutritional status, physical activity, pulmonary and radiological findings [14]. A composite score (CF score) was calculated by summing up all four scores; a score of >80 suggested mild disease and <40 an advanced disease.

Approximately 5 mL of blood sample in EDTA was collected from each patient and DNA was extracted by standard phenol–chloroform (for stored samples) [15] or by salting-out (for fresh samples) method [16].

2.1. Mutation analysis

Initially, all patients were tested for p.F508del and c.3849+10 kb C>T mutations. The samples which were negative for these two mutations were analysed by single strand conformation polymorphism/heteroduplex analysis (SSCP/HA) of 19 out of 27 exons of the *CFTR* gene, in which mutations were commonly seen (exons 3, 4, 7, 10, 11, 12, 13A, 13B, 19, 20, 21 and 2, 5, 9, 14a, 14b, 16, 17a, 17b and 23) [11]. The samples that showed a shift in the band pattern were sequenced. For p.F508del, a 50 base pair region flanking this deletion was amplified by PCR using a previously published protocol and run on 10% PAGE [17]. The analysis of c.3849+10 kb C>T was done by amplifying 437 bp region in the intron 19 and restriction with *Hph* I [18]. PCR for SSCP/HA was carried out in 25 μL volumes. For fragments which were large, restriction digestion was carried out.

3. Results

3.1. Demographic profile of the patients

The origin of patients could be traced to almost all states in north India. The maximum number (23) of patients had their origin from the state of Uttar Pradesh (Fig. 1). The other common place of origin was the neighbouring country of Pakistan with history of migration to India (16), followed by the states of West Bengal (10) and Delhi (8). The place of origin of the father and mother was different for five patients.

The patients were predominantly male (62 males and 38 females). Consanguinity was present in 13 families. History of a confirmed case of CF in the family was present in two cases. Clinical history, highly suggestive of CF in a previous child but not proven, was present in 26 patients.

The mean (±SD) and median (IQR) age at diagnosis were 52.7±60.2 and 30 (8.0–72.0) months respectively although more than half of the patients (54) had developed at least one symptom suggestive of CF by three months. One patient was diagnosed in the first week after birth. The oldest age at diagnosis was 28 years. The mean age at onset of symptoms was 10.7 months and the median age at onset of symptoms was 2.8 months. Thirty-four patients were diagnosed within the first year of life and 96 patients were diagnosed by 16 years.

3.2. Clinical profile

3.2.1. Symptoms and CF score

A total of 98 patients had recurrent or persistent pneumonia at the time of presentation. Malabsorption was present in 85/97 (88%) and failure to thrive (FTT) in 95/98 (97%) of patients. Hepatomegaly (>2 cm) was present in 18/91 (20%). The mean CF score calculated was 49±15.8, indicating advanced disease at the time of diagnosis. Eighteen (20%) patients had evidence of advanced disease with a CF score of <40. There were 42 patients with a score of 40–60 and 32 patients with a score of >60. Clubbing was present in 66/97 (68%) of patients.

3.2.2. Anthropometry and nutritional status

Physical examination revealed malnutrition in 72/82 (88%) of patients (Indian Academy of Paediatrics classification) in whom detailed anthropometry was available. Grade I, II, III and IV malnutrition was seen in 18.5, 25.6, 24.4 and 19.5% patients respectively.

3.3. Laboratory findings

The mean sweat chloride was 97.6 mEq/L in 93 patients tested. Sweat chloride could not be analysed in a few patients due to poor sweat weight or advanced disease state. *Pseudomonas* spp was cultured from respiratory secretions of 45/97 (46%) patients and *Staphylococcus* spp in 11/96 (11%). Other laboratory tests such as blood counts, liver function tests, chest X-rays, CT chest, ultrasound of abdomen and fat malabsorption studies were performed on individualised basis at the time of enrolment and during follow up.

3.4. Mutation analysis

Testing for p.F508del, c.3849+10 kb C>T and SSCP/HA of 19 exons of the *CFTR* gene in the 100 CF patients (i.e. in

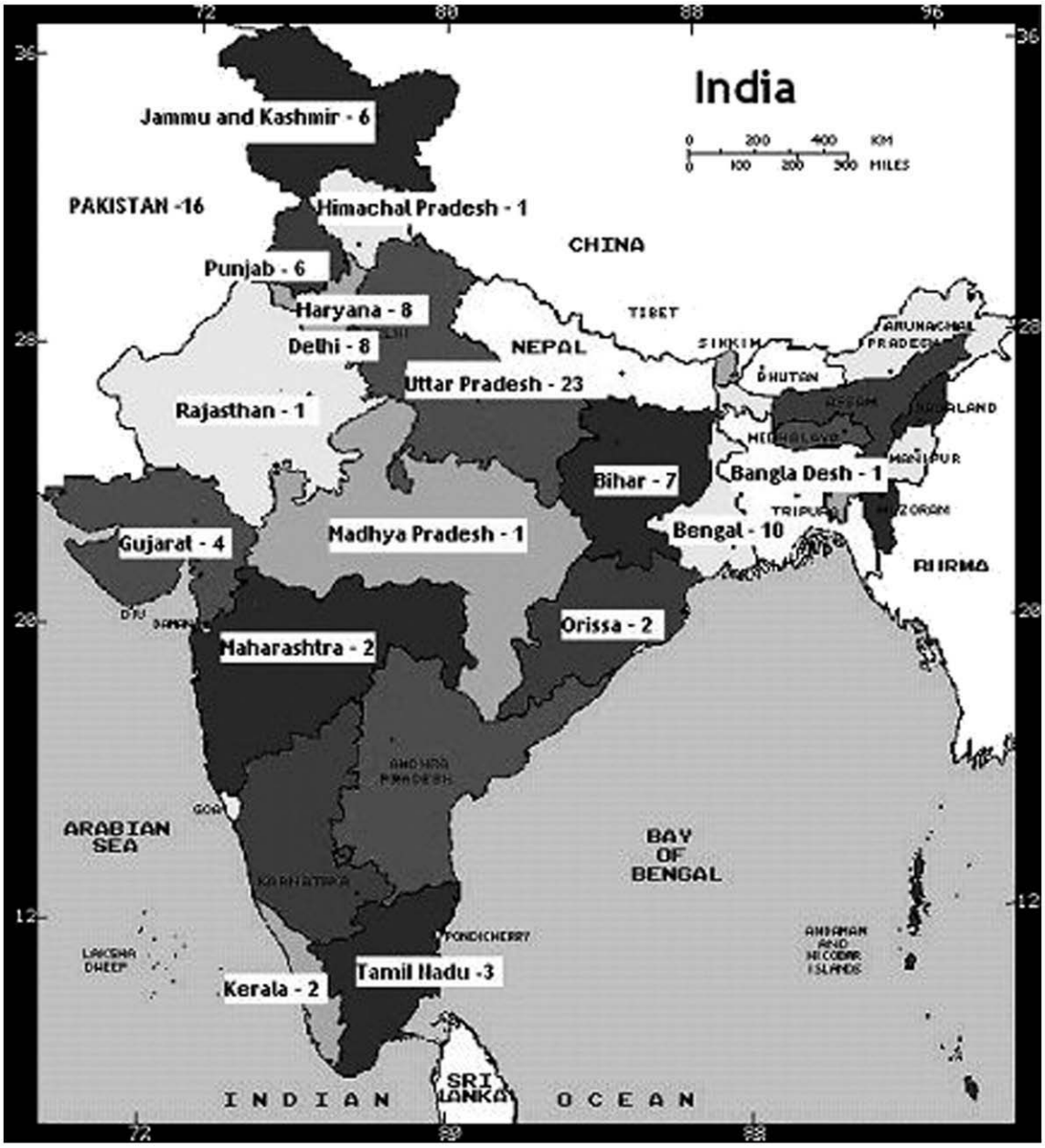

Fig. 1. Map of India showing the area of origin of patients.

200 chromosomes) identified at least one mutation in 40 patients (66 chromosomes) (Table 1). Out of these 22 were homozygous for a specific mutation. The most common mutation was p.F508del, identified either in homozygous or heterozygous condition in 33 (33%) patients and 53 (26.5%) of chromosomes. Three other mutations, c.3849+10 kb C>T, c.1161delC and p.S549N were seen in two patients each. Frequency of other mutations did not exceed 1%.

3.4.1. SSCP/HA

SSCP/HA identified at least one mutation in exons 3, 4, 7, 11 and 19 viz. p.R75Q in exon 3, p.G149X in exon 4, c.1161delC, p.R352Q and c.1002-7_1002-5delTTT in exon 7, p.S549N in exon 11 and p.R1158X in exon 19. A mutation (p.L183I) was detected in exon 5.

3.4.2. Novel mutations identified

Three mutations (c.1002-7_1002-5delTTT, p.L183I, p.G149X) unpublished so far (Table 2) were detected in one patient each. While c.1002-7 to 1002-5delTTT was detected in homozygous state, p.L183I and p.G149X were detected in heterozygous state. Reports of these mutations have been submitted to the Cystic Fibrosis Genetic Analysis Consortium.

Table 1
Genotype of the 100 patients with cystic fibrosis

Mutation	Number of patients ($n=100$)
p.[F508del]+[F508del]	20
p.[F508del]+[?]	9
p.[F508del]/[c.3849+10 kb C>T]	2
p.[F508del]+[R1158X]	1
p.[F508del]+[G149X]	1
c.[1002-7_1002-5delTTT]+ [1002-7_1002-5delTTT]	1
c.[1161delC]+[1161delC]	1
c.[1161delC]+[?]	1
p.[S549N]+[?]	2
p.[L183I]+[?]	1
p.[R352Q]+[?]	1
[?]+[?]	60

3.5. Genotype–phenotype correlation

On comparing patients with p.F508del (both homozygous and heterozygous) and others patients (both mutation positive and negative), there was a significant difference observed in the age at onset (2.3±2.6 vs. 14.5±22.4 months; $p=0.000$), age at diagnosis (40.9±76.9 vs. 58.2± 50.4 months; $p=0.006$) and CF score (40.8±13.8 vs. 52.1± 15.5; $p=0.02$), all being lower in p.F508del positive patients. The mean sweat chloride level (107.7±20.5 vs. 93.5± 30.6 mEq/L; $p=0.013$) was higher in p.F508del positive group.

The new mutations showed a clinical profile similar to classical CF. Severe clinical phenotype was seen in a patient with p.G149X mutation and low CF score, whereas milder CF phenotype was seen in the two patients with c.1002-7_1002-5delTTT and p.L183I. The patient with c.1002-7_1002-5delTTT mutation had predominantly gastrointestinal symptoms and lower respiratory tract infections were not present.

It was interesting to note that the majority of patients with p.F508del originated from Pakistan. Out of 33 patients who had p.F508del on one or both chromosomes, 17 patients originated from Pakistan and neighbouring states of India namely Jammu and Kashmir, Punjab and Gujarat. Follow-up was available in 80 patients. Nine patients had expired during follow-up. Of these six were p.F508del homozygotes, one was compound heterozygous for p.F508del with p.R1158X and another was homozygous for c.1161delC. One patient whose mutations were not characterised also died during follow-up.

4. Discussion

4.1. Demographic profile

CF was not confined to any particular geographic area of India. Consanguinity was present in 13% of the patients. This is similar to earlier reports from India [19,20] but less than that reported (50%) by Spencer et al. [7] from Indian subcontinent patients in UK. Out of the 13 patients born to consanguineous parents, eight were homozygous (7 for p.F508del and 1 for c.1161delC) and one was heterozygous (for c.1161delC); suggesting consanguinity results in a higher risk of homozygous CF mutations in India. Male preponderance observed in this study (male: female ratio of 1.6:1) may be due to the preferential treatment given to male children in the society.

4.2. Clinical profile

The mean age at diagnosis (52.7 months) was much higher than the mean age at diagnosis of CF children in USA, which is 6 months. The mean age at onset of symptoms was 10.7± 19.5 months and the median was 2.8 (1–9) months in this study. A wide gap between onset of symptoms and diagnosis highlights the need for a better awareness programme about CF in India. The clinical features recorded in this study were consistent with the classical CF phenotypes described [9,14,21,22]. The clinical scores of patients (mean 49±15.8) suggest an already advanced disease at the time of diagnosis, probably related to delayed diagnosis.

4.3. Laboratory investigations

The mean sweat chloride level (97.6±28.7 mEq/L) was similar to earlier reports from India [19,23]. The frequency

Table 2
New mutations identified in patients with cystic fibrosis

	Mutation 1	Mutation 2	Mutation 3
Exon/intron	Exon 4	Intron 6b	Exon 5
Mutation	p.G149X (c.577G>T)	c.1002-7_1002-5delTTT	p.L183I (c.679C>A)
Resulting change	Stop codon at 149	Deletion of 3 bp in intron 6b/exon7 boundary	Leucine to isoleucine at 183
Genotype of the patient	p.[F508del]+[G149X]	c.[1002-7_1002-5delTTT]+[1002-7_1002-5delTTT]	p.[L183I]+[?]
Gender	Male	Female	Female
Age at diagnosis (months)	2	54	84
LRTI[a]	Yes	No	Yes
Malabsorption	Yes	Yes	Yes
CF score	30	80	60
Sweat chloride (mEq/L)	98[b]	200, 47	57, 60

[a] LRTI — Lower respiratory tract infections.
[b] Sweat chloride was done only once.

of different morphotypes of *Pseudomonas aeruginosa* and antibiotic resistance is higher in Indian children with CF [23].

4.4. Mutation profile

Out of 200 chromosomes tested, mutations could be identified in 66. In a previous study of 24 patients from our centre with similar methodology as in the present study, at least one mutation could be identified in 75% of patients [11]. A review of worldwide analysis of *CFTR* mutations [24] has shown that the rate of mutation detection varies from a maximum of 100% in Belgium to a minimum of 33.3% in Venezuela. The mutation detection rate was higher in patients originating from Pakistan and neighboring states of India. WHO has estimated the mutation detection rate in Indian CF patients to be about 50–59% [25]. In a subgroup of patients studied by McCormick et al. in UK, with data for 27 common mutations, at least one mutation could be identified in 9 out of 12 Indian patients, 32 out of 52 Pakistani patients and 3 out of 6 Bangladeshi patients [26].

In a compilation of data on mutations in Asian patients in the US, mutations were identified in 50% of patients [6]. In another study mutations were identified in nearly 86% (22 out of 26 patients) of patients with Pakistani origin, of which 15 were homozygous [27].

The low mutation detection rate in our study could be due to low sensitivity of SSCP under the conditions we have used. Secondly, although the exons selected carried majority of the mutations that have been reported, the mutations in our CF patients could be residing in other exons or introns of the gene, which were not screened.

4.4.1. Novel mutations

c.1002-7_1002-5delTTT results in the deletion of 3 thymidine at the acceptor site of intron 6b/ exon 7 boundary. This mutation in homozygous condition was seen in a 54-months old girl who originated from Punjab and was admitted in the paediatric ward. She had predominantly gastrointestinal symptoms.

p.G149X is a nonsense mutation resulting from the substitution of guanine by thymidine at the nucleotide position 577. This transversion changes the codon 149 from GAA to UAA resulting in replacement of the amino acid 149 from glycine to stop codon. This is an amino acid present in the hydrophobic transmembrane domain of the CFTR. This mutation was identified in a in a boy at two months of age. He was compound heterozygous for p.F508del with p.G149X and had meconium ileus, lower respiratory tract infections (LRTI) and severe FTT.

p.L183I is a substitution of cytosine by adenosine at the nucleotide position 679, which changes the amino acid 183 to isoleucine from leucine. This amino acid is also a part of hydrophobic transmembrane domain. This was a 7-year old girl with LRTI, sinusitis, malabsorption and FTT. Her other mutation has not been not identified yet.

4.5. Genotype–phenotype correlation

The phenotypes of the patients in the present study were similar to that described in literature [21,22]. Other studies have not found a significant difference in the phenotype of patients with different genotypes except for few mutations such as c.3849+10 kb C>T [28]. This may be due to early diagnosis and optimal treatment in the Western countries. Most of the children in this study were not getting appropriate enzyme replacement at the time of diagnosis and even after diagnosis, which caused severe malnutrition and a severe disease in them.

This study has shown that there is a considerable difference in the mutation spectrum of CF patients originating from Pakistan and the neighbouring states of India. Half of the CF patients from this region are either homozygous or heterozygous for p.F508del while only one in six CF patients from the rest of India is p.F508del positive.

Since p.F508del is the commonest mutation in our CF population, any national strategy for mutation screening in CF must start with testing for p.F508del. Simple PCR-based methods are already in wide use for the identification of this mutation. This should be followed by screening for the other three common mutations, p.S549N, c.3849+10 kb C>T and c.1161delC identified in this study. PCR-based identification of common mutations is available [18,29] and new methods are being continually developed. When the mutations are not identified in the index child, prenatal diagnosis may be performed using dinucleotide repeat markers, which are generally informative [30,31]. Despite some limitations, the present study has managed to answer some of the questions related to CF mutations in Indian CF patients.

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